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ORIGINAL ARTICLE

Planting density and culture time of wheat seedlings affect their growth rate and exometabolite production

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Abstract The effect of wheat seedling planting density (from 25 to 300 seedlings/70 cm^2) and culture time (from day 1 to day 3 in water culture) on quantitative and qualitative composition of root exudates and root growth rate was studied. The number of root border cells per root was assessed. Amount of total protein, high-molecular weight proteins, and carbohydrates, as well as protease activity in root exudates were evaluated. The "root-microenvironment" system was found to be a highly dynamical nonlinear system being a function of the culture duration, planting density and special aspects of transformation of extracellular exometabolites. During culture from day 1 through day 3, variability of the measured parameters increased.

Keywords Wheat seedlings · Root exudates · Root exometabolites · Planting density · Border cells

Introduction

The root system of higher plants can become a good and promising target for modern biotechnology (Smetanska et al. 2012; Gleba et al. 1999). Roots demonstrate relatively fast growth and may be cultured in a variety of media; in addition, they have a unique excretory system which creates a specific microenvironment (Bais et al. 2001).

The root excretory system is represented by several elements, including somatic root cells; root border cells, which are detached from the root surface and continue to function in the gel cap; and a variety of macromolecules. These macromolecules, especially proteins, amino acids and polysaccharides, form the rhyzosphere (Wen et al. 2007; Baetz and Martinoia 2014). It was shown that root excretory system is being formed during the period from day 1 to day 3 of root growth, and experimental removal of the microenvironment induces its rapid restitution (Wen et al. 1999). The wheat exometabolites can be used as biologically active compounds exhibiting a wide range of physiological actions (Naumov et al. 1993; Bozhkov et al. 1996).

Despite the high potential of root biotechnologies, no attempt to develop the technology of industrial production of root exudates has been successful so far. One of the main challenges encountered in the development of such technologies is a lack of consistency in the amount of the obtained product throughout the root growth. Therefore, investigation of the effect of various modes of culture on the qualitative and quantitative composition of root exudates can help to overcome this problem.

Previously, it was hypothesized that the root and its surrounding microenvironment form an integrated self-regulating system (Naumov et al. 1993). The study of mechanisms of self-regulation in the root-microenvironment system is of interest no only in terms of mechanisms of plant growth and development regulation and formation of plant and bacterial communities, but also in terms of production of biologically active compounds for the pharmacy, cosmetology and medicine (Haichar et al. 2014). Along with this, we consider the root-microenvironment

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system to be a convenient research model to study dynamics of the nonlinear behavior of biological systems.

One of the approaches leading to understanding of self-regulation in the root-microenvironment system, is to study the effects of seed planting density on the rate of exometabolites excretion, composition of exudates and root growth rate. As planting density in hydroponic culture affects physical and chemical properties of the medium, and the microenvironment affects growth and function of the root, it may represent a natural mode of regulation of root exometabolite production.

In the present study we were investigated the effect of planting density on the volume of root exudates, protein and carbohydrate content, activity of proteolytic enzymes, and possible relationship between these parameters and the number of root border cells.

Materials and methods

Plant material

Seedlings of soft winter wheat (*Triticum aestivum* L.), cv. Donetskaya-48 were used in the study. The seeds were soaked in water for 2 h, then incubated in $KMnO_4$ for 10 min, and soaked in distilled water in a flat glass container at 25 °C for 21–22 h. The germinating seeds were transferred into Petri dishes (25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350 seeds per dish). Sterile distilled water was added to each dish and seeds were cultured at 28 °C and 24 h light with intensity of 5 klx. Twenty-four hours later root border cells were counted.

Collection of root exometabolites

The conditioned culture medium containing root exudates was transferred from Petri dishes into test tubes, and seedlings in the dishes were additionally washed with sterile distilled water. The conditioned culture medium was used to analyse exometabolites in root exudates, including total protein and carbohydrate content, and protease activity.

Root border cell counting in situ

The roots of 1-day-old wheat seedlings were fixed in 2% glutaraldehyde and stained with 0.06% Trypan Blue. Then, apical portions of the roots were placed in a drop of water on a glass slide, covered with a cover glass, and the number of root border cells in the apex zone was counted under light microscope (LOMO, Russia) and expressed as the number of root border cells per root.

Preparative isolation of root border cells

For preparative isolation of root border cells, 0.5 ml of distilled water was added into a compartment of a plastic culture plate fixed on a magnetic stirrer. The apical root zone of 1.5–2.0 cm long was submerged into vigorously stirring water for 1–2 min in order to remove the apical root gel cap with root border cells. Twenty to 50 roots were washed out in one compartment. Washing medium was transferred from the compartment into test tubes and centrifuged at 3000 *g* for 15 min in order to sediment root border cells. The pellet containing root border cells was fixed with 2% glutaraldehyde and stained with 0.06% Trypan Blue. The number of root border cells removed by the preparative isolation method was counted in Goryaev haemocytometer and expressed as the number of cells per root.

Evaluation of the effect of planting density of wheat seedlings on root growth and root exometabolite excretion

To evaluate the effect of planting density on root growth rate and root exudate composition, seeds were placed into Petri dishes (25, 50, 100, 150, 200, 250, 300 and 350 seeds per dish). Collection of root exudates and root length measurements were carried out on day 1, 2 and 3 upon the beginning of culture.

Evaluation of total protein content

The total protein content in root exudates was determined by the Lowry assay (Lowry et al. 1957).

Evaluation of high-molecular weight protein content

High-molecular weight (HMW) protein was precipitated from the soluble fraction of exudates with 76% ethanol for 12 h at 4 °C. The precipitate was then dried and dissolved in 1 N NaOH, and protein was assayed in the aliquots by the Lowry method (Lowry et al. 1957).

Evaluation of total carbohydrate content

The carbohydrate content was determined according to Molisch (Gerhardt 1983). The amount of total carbohydrates was calculated using a standard calibration curve obtained for glucose and was expressed as mg glucose/100 seeds.

Evaluation of protease activity in root exudates

The protease activity in the soluble fraction of exudates was determined by a standard method (Yermakov et al. 1987).

Statistical analysis

The data on root length, amount of proteins and carbohydrates in root exudates for each category of planting density and culture time are presented as mean values of five replicates. In a table and figures the data are presented as mean ± standard error of the mean (SEM). Group comparison was performed by Mann–Whitney test ($p < 0.05$). The strange attractors for root length and protein content were built using the time delay method with STATISTICA 6.0 software package.

Results and discussion

Morphologic characteristics of the root-microenvironment system

After 24 h of growth, wheat roots were 0.7–1 cm long, and the rhizosphere was already formed (Fig. 1a). In the root-microenvironment system, the rhizosphere is characterized by well-developed border cells, as well as by the presence of a well-structured zone rich in polysaccharides and other macromolecules (Fig. 1b). It was shown that border cells play an important role in formation of the root microenvironment by excreting a variety of compounds in the surrounding medium (Doornbos et al. 2012).

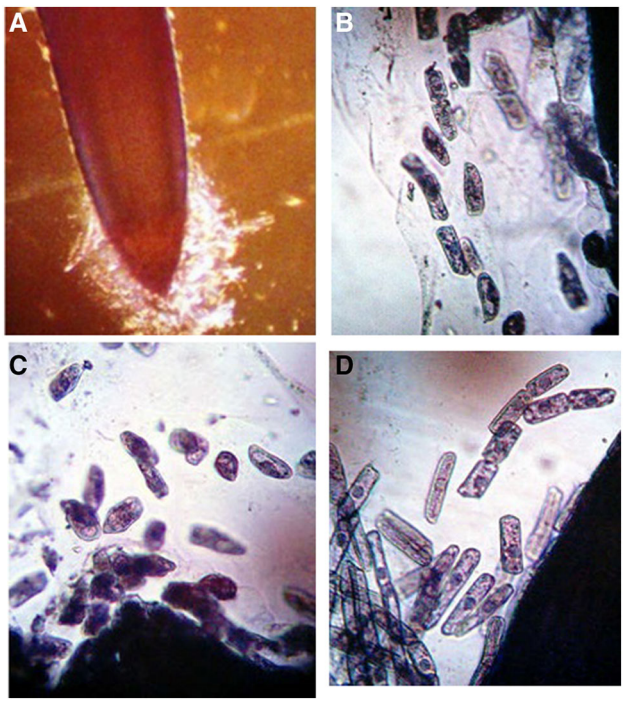

Fig. 1 Morphology of the root apex of the 1-day-old seedlings: **a** root apex; **b** gel cap with root border cells located in polysaccharide mucilage; **c** round border cells present near apex surface; **d** individual and aggregated oval border cells near lateral apex surface. Arrows indicate the borders of the polysaccharide mucilage. Expressed heterogeneity of the root microenvironment is observed

Table 1 Root border cell number in apex of wheat seedlings evaluated in situ and by preparative method day 1 to day 3 of growth

Day of growth	Root border cells (n per root)	
	In situ	Water wash
1	34 ± 3	67 ± 8
2	41 ± 4	87 ± 7
3	40 ± 3	85 ± 6
Mean	38 ± 2	79 ± 6
LSD ($p \leq 0.05$)	14	28

The data is presented as means of five independent experiments with standard errors of the mean

The number of border cells in the wheat seedlings at day 1 was not high: 30–40 cells per root, and this number did not change by day 3, when counted in situ. The root border cells released into the rhizosphere, had an elongated shape, while the cells at the root apex were oval (Fig. 1c). Some border cells formed cell aggregates (Fig. 1d).

To assess the strength of intercellular contacts between the border cells and other cells of the root, directed washing with water was used. It was found that washing with water increased the number of root border cells in the rhizosphere up to 80–90 cells, i.e. twofold (Table 1). In this case the cells were arranged into aggregates (Fig. 1d).

In summary, the root contains a population of cells that can be mechanically transferred to the rhizosphere and can be referred to as border cells. A population of border cells contains both individual cells and cell aggregates. The intercellular contacts between border cells can be quite strong and do not break down due to washing with water, while root border cells can be easily separated from other root cells this way.

The number of root border cells in the apex of wheat seedlings from day 1 to day 3 of growth increased only by 25–30% (Table 1). Analysis of the main components of wheat seedling root exudates showed that they are comprised of approximately 65% of carbohydrates and over 35% of proteins (Fig. 2).

Effect of seed planting density on the root growth rate

The root length reached 1.3–1.6 cm by the end of day 1 of culture, regardless of the seed planting density (Fig. 3). It should be noted that root growth rate at the low density of 25–50 seeds/70 cm^2 was lower than at the density of 100 seeds/70 cm^2. In 2-day-old seedlings (at day 2), the variability of this parameter in all density groups was higher compared to 1-day-old seedlings; and root length was from 2.4 to 3.5 cm.

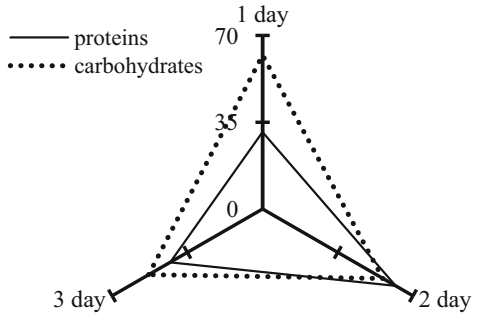

Fig. 2 Amount of proteins and carbohydrates in root exudates of 1-, 2- and 3-day-old seedlings. Mean values of five independent experiments are shown

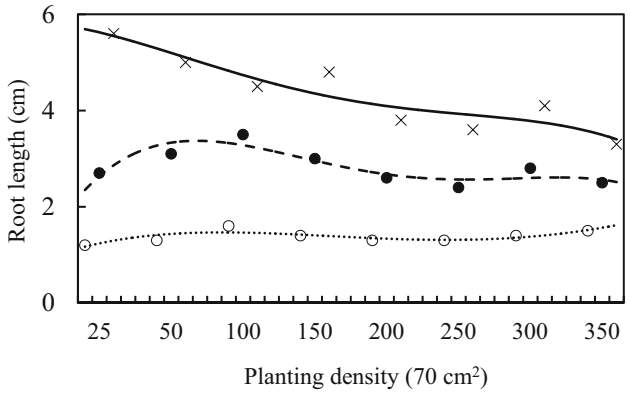

Fig. 3 Root length of 1-, 2- and 3-day-old seedlings at the different planting densities. Bars represent mean ± SEM of five replicates

The seedling root length was even more variable in 3-day-old roots. At the same time, there was a negative relationship between the root length and planting density (Fig. 3): the root length was the highest (5, 6 cm) at the planting density of 25 seeds/70 cm^2 and the lowest at the density of 350 seeds/70 cm^2 (3, 3 cm) (Fig. 3), with the mean difference of 2.3 cm.

Therefore, the wheat root growth rate during the first 24 h of growth remained unchanged within the density range from 25 to 350 seeds/70 cm^2, while after 48 h of growth, there relationship between the root length and planting density was described with an S–shaped curve, and after 72 h of growth, there was a negative relationship between the root length and planting density. This means that the growth of roots during the first day of culture did not depend on the density of seedling planting within the range from 50 to 350 seeds/70 cm^2, but by the end of day 3 of culture, a negative relationship between the density of planting and the root growth rate was observed.

To analyze this non-linear system, we performed phase space reconstruction using the time delay method. This approach allows characterization of non-linear systems according to their potential stability. In terms of stability, the systems can be divided into systems with broad-range stability, i.e. systems demonstrating resistance against a lot of factors, and systems with a narrow-range stability, i.e. those resistant only to some environmental factors.

The calculation results presented in Fig. 4, strongly suggest that the 1-, 2- and 3-day-old seedlings have different phase portraits. While for the 1-day-old seedlings all points lie in a very narrow area, i.e. are attracted to a singular point, in the 2-day-, and especially in the 3-day-old seedlings experimental points have several centers of attraction (Fig. 4). Consequently, the measured parameters in the 1-day-old seedlings growing in water culture remain stable under a broad range of factors, while these parameters in 2-day-, and especially, in 3-day-olds seedlings are characterized by resistance under a narrow range. This, in turn, suggests that the water culture of the 1-day-old seedlings is homogeneous and its parameters are minimally affected by the environment, which makes it resistant and stable.The water culture of the 3-day-old seedlings is heterogeneous and sensitive to a variety of factors, and the water culture of the 2-day-old seedlings occupies an intermediate position between these two.

Another important consequence of the results obtained, is that this system is most sensitive to the influence of regulatory factors for 2-day-old seedlings, as at this age a variety of points of attraction or directions of metabolic patterns is being formed. In this state, the system is most responsive to exogenous environmental factors. Therefore, using different culture time and planting density, the seedlings growth rate and exometabolic composition can

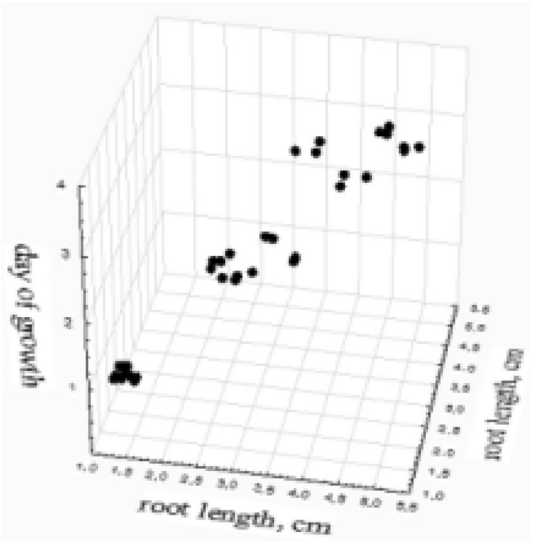

Fig. 4 Attractors for root length of 1-, 2- and 3-day-old wheat seedlings at different planting densities

be regulated. This can be indicated by an expanding corridor on the phase space (Fig. 4).

It is necessary to understand how and why a closed system, into which no additional components are introduced, and from which nothing is removed, passes such nonlinear stages of development. The non-linear changes in the root length, manifested in the increase in individual variability from day 1 to day 3, may be due to non-linear mechanisms. One of such mechanisms is associated with the dynamics of the qualitative and quantitative composition of exudates, and consequently can change physical and chemical properties of the root microenvironment. Components of the microenvironment can influence the growth rate of roots in water culture (Bozhkov et al. 2007).

Effects of planting density and culture time on the root exudate composition and rate of excretion of proteins and carbohydrates

In the following experiments, the rate of protein excretion in culture medium at different densities of seedling planting was determined.

It was shown that during the first 24 h of growth at the density of 50 seeds/70 cm^2, roots excreted 1.8 mg protein/100 seedlings, and during the same period of time but at the density of 100 seeds/70 cm^2, the rate of excretion was 3.6 mg of protein/100 seedlings. By increasing the density up to 200 seeds/70 cm^2, the rate of protein excretion decreased down to 1.3 mg/100 seedlings and reached plateau at that level (Fig. 5). After 2 days of culture at different planting densities, protein content in water medium slightly differed from that on the first day of growth, but it was more dependent on the density of planting (Fig. 5). Protein content in exudates after 3 days of culture increased abruptly compared to 2 days. There was a 7.3-fold increase in protein content at planting density of 50 seeds/70 cm^2, whereas with further increase of planting density, the excretory rate decreased, except for the density of 250 seeds/70 cm^2. The obtained results indicate a non-linear relationship between these two parameters (Fig. 5).

Therefore, the specific rate of protein excretion in water medium during the root growth from day 1 to day 3 was non-linear: there was a drastic increase from day 2 to day 3 of growth, and the rate of excretion depended on the density of seedling planting.

Since total excreted proteins comprised a wide range of molecules with different molecular weight, the amount of high molecular weight proteins (HMWP) (10–12 kDa) was evaluated in the next experiments.

The of proteins with the molecular weight of more than 10 kDa comprised from 20 to 70% of the total protein content and this parameter was a significant extent associated with the time of culture. While after 1 day of culture there were from 22 to 50% of high molecular weight proteins, on day 2 the macromolecular proteins comprised 50–70% (Fig. 6).

There was a 2–3 time decrease in the amount of high molecular weight proteins on day 3 compared to the amount measured after 2 days of culture, regardless of the density of planting. It should be noted that the decrease in the high molecular weight proteins at day 3 was accompanied by increase in the excretion of total proteins. These results can be explained by the change in composition of the excreted proteins during the root growth and/or by the breakdown of high molecular weight proteins in the exudates.

In the following experiments, total protease activity in the root exudates was analysed. It was found to be the highest in the 1-day-old seedling exudates. Protease activity was the highest at the lowest planting density,

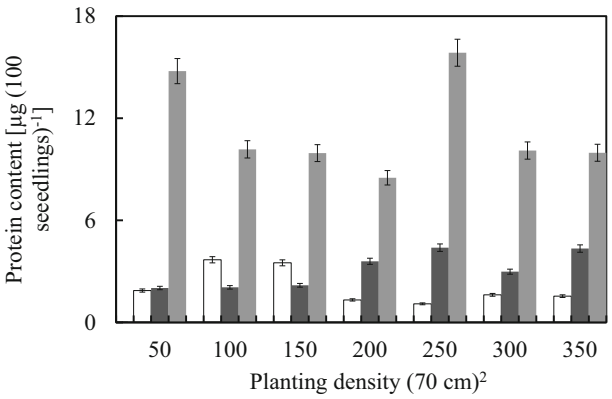

Fig. 5 Total protein content in root exudates of 1-, 2- and 3-days-old wheat seedlings at different planting densities. Bars represent mean ± SEM of five replicates

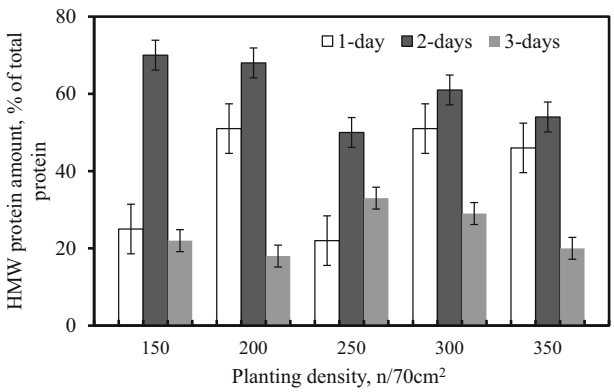

Fig. 6 High-molecular weight protein (HMWP) content (% of total protein) in root exudates of 1-, 2- and 3- day-old seedlings at different planting densities. Bars represent mean ± SEM of five replicates **a** ELECTROPHOREGRAM of HMWP: paths 1 and 5 are proteins with known molecular masses; paths 2–4 are proteins of exudates of 1-, 2- and 3-days-old seedlings, respectively (**b**)

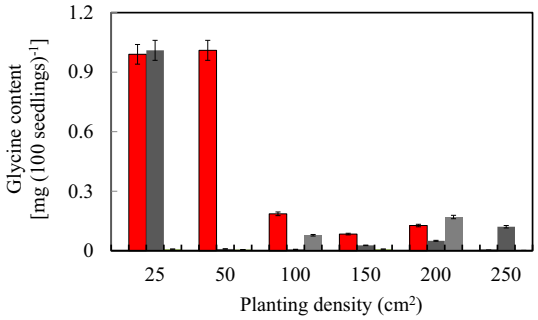

Fig. 7 Protease activity (mg glycine/100 seedlings) in root exudates of 1-, 2- and 3-days-old wheat seedlings at different planting densities. Bars represent mean ± SEM of five replicates

while it was several times lower or absent at higher planting densities (Fig. 7). In the exudates from 2–3-day-old seedlings, protease activity was not detected, or it was extremely low (Fig. 7).

Therefore, presence of protease activity in exudates of seedlings suggests possible changes in the rhizosphere protein composition due to the enzymatic hydrolysis. Since there is no strong relationship between the amount of high molecular weight proteins and protease activity, it can be assumed that the observed differences in the composition of high molecular weight proteins were caused by other factors.

The amount of carbohydrates in root exudates was shown to be much more strongly associated with the density of seedling planting. At the density of 25 seeds/70 cm^2, the carbohydrate amount in exudates of the 1-, 2- and 3-day-old seedlings was 1.8, 5.5, and 12.2 mg glucose/100 seedlings, respectively. The highest variability in the amount of carbohydrates in root exudates of 1-, 2- and 3-day-old seedlings was observed at planting density of 300 seeds/70 cm^2 (Fig. 8).

It can be suggested that there is a strong non-linear relationship between the change in the qualitative and

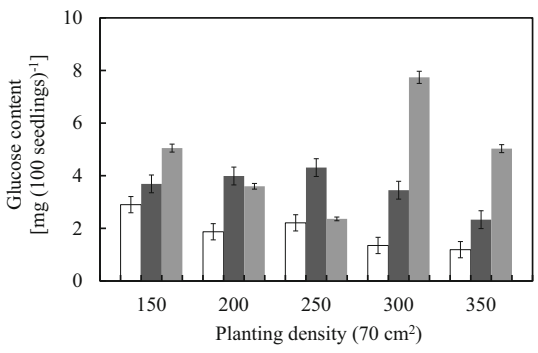

Fig. 8 Carbohydrate content (mg glucose/100 seedlings) in root exudates of 1-, 2- and 3-days-old wheat seedlings. Bars represent mean ± SEM of five replicates

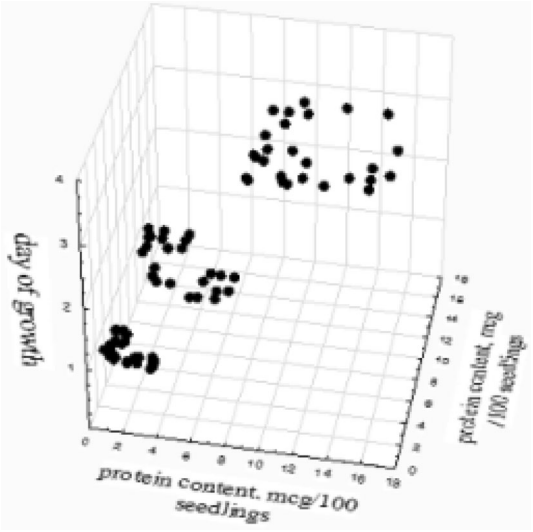

Fig. 9 Attractors for protein content in root exudates of 1-, 2- and 3-day-old wheat seedlings at different planting densities

quantitative composition of exudates, planting density and culture time.

Presentation of the amount of proteins in exudates as a function of the planting density and time of culture on the phase space portrait plot, showed regular behaviour of this parameter depending on the root growth rate (Fig. 9).

Previously, it was shown that during root growth, changes occur not only in qualitative but also in quantitative composition of exudates, as well as in physical and chemical characteristics, such as pH, electromotive force, and viscosity (Bozhkov et al. 1996), which further suggests that the root-microenvironment system is non-linear and a highly dynamic.

An additional mechanism of development of the non-linear behaviour in the root-microenvironment system is associated with inhibition of excretion upon accumulation of exometabolites, and, conversely, to its stimulation in case of the removal or destruction of the exudate components in the medium i.e., their extracellular metabolism (Bozhkov et al. 2009).

The data obtained suggest that the development of the root biotechnologies can be performed at least in several strategic areas:

1. To obtain exudates with a relatively constant product composition, but when only low volumes are required, 1-day-old seedlings should be used;
2. To obtain large quantities of exudates, 3-day-old seedlings should be used, optionally with further purification of the target product;
3. If regulation of the of the qualitative and quantitative composition of exudates must be included in the process, 2-day-old seedlings should be used;

4. The root microenvironment is a multi-component system which includes not only proteins, carbohydrates, amino acids and other molecular components, but also the root border cells,
5. The non-linear functioning of the "root-microenvironment" system can be explained by different time-related dynamics of excretion, by extracellular metabolism of these components on one hand and the influence of exometabolites on the root metabolism and subsequent excretion on the other hand.

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